LARGE SAMPLE VOLUMES IN PREPARATIVE CHROMATOGRAPHY

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SUMMARY

Equations are developed which relate output profiles resulting from large sample input volumes to the well-known expressions for elution chromatography. The sample volumes are such that output profiles are intermediate to the usual Gaussian curves resulting from very small sample volumes, and frontal output curves described by an integral Gaussian equation. Simple expressions which describe the curve height at the maximum and curve width as a function of input volume are presented. This information is used to relate the apparent plate height to the plate height for small sample volumes and variation in observed resolution (R_S') as a function of sample volume. It is demonstrated that $1/R_S' = (1/R_S) + (v/4V_R)$. The equations are tested by reversed-phase high-performance liquid chromatography and preparative chromatography with silica as a stationary phase.

INTRODUCTION

Recent technological achievements which have led to improved analysis by high-performance liquid chromatography (HPLC) are now being applied to preparative separations. DeStefano and Kirkland[1] discussed preparative HPLC and included in their recommendations the use of relatively large sample volumes. Other current work in this field includes that of Wehrli *et al.*[2] and Scott and Kucera[3].

Some of the earliest theoretical work in chromatography was concerned with feed volume and its relationship to the position of the maximum and the width of the eluted solute profile. Porter *et al.*[4] demonstrated that the definite integral of the Gaussian distribution could be used to calculate the elution curves for finite sample volumes. They showed that the elution volume at which the observed maximum occurs, V_{max}, could be related to the sample volume through the expression

$$V_{max} = V_R + v/2 \quad (1)$$

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where v is the sample volume, and V_R, the retention volume, which results as v approaches zero. They also pointed out that "the apparent number of plates in a column calculated by the procedure frequently used, which does not take into account sample size, is dependent upon the sample size." Later discussions of the effect of sample volume[5] on chromatograms have been from the perspective of non-ideal interactions and the errors that are introduced into calculations of retention data and partition coefficients by inefficient injectors and injection techniques.

However, many investigators by design have used large sample volumes. These include Spitz *et al.*[6], who made use of frontal outputs for preparative separations by classical liquid chromatography, Rothbart *et al.*[7], who explored some of the related mathematical relationships in countercurrent distribution, and Barford *et al.*[8], who utilized the approach for preparative fractionation by countercurrent distribution. The use of large sample volumes in gas chromatography and the related use of multiple inputs in countercurrent distribution have been described by Reilley *et al.*[9]

In this report we demonstrate that there are straightforward relationships between the observed output profiles resulting from large sample volumes in preparative chromatography and the almost infinitesimal sample volumes used for analytical separations.

THEORETICAL

Under near ideal conditions and with small solute input volumes, chromatographic curves are usually described by a Gaussian expression. When a very large volume of solute solution is introduced into a column, a frontal or breakthrough profile is produced similar to that depicted in Fig. 1a. The leading section of the solute profile may be described by the integral of the Gaussian or eqn. 2[7].

$$Y = \frac{M}{M_F^*} = \text{erf}\,[(V - V_R)/\sigma] \tag{2}$$

Here M_F^* is the sample concentration in the feed solution, M is the sample concentration at output volume V, and σ is the standard deviation of the Gaussian curve. The retention volume, V_R, for a frontal output occurs at $Y = 0.5$. Wehrli *et al.*[2] noted that a feed volume about 5 times σ is necessary to just achieve a step function output of the sort depicted in Fig. 1a. The feed volume required can be specified exactly if an acceptable value of Y is stated (Table I)[7,8].

TABLE I

VOLUME OF FEED REQUIRED TO PRODUCE A FRONTAL OUTPUT AT VARIOUS ACCEPTABLE LEVELS OF $Y < 1$

Acceptable Y	*Feed volume required*
0.977	4.00 σ
0.991	4.70 σ
0.994	5.00 σ
0.999	6.00 σ

Any volume of feed solution in excess of that required to produce a frontal output may be considered merely to extend the length of the plateau. For a series of compounds whose elution curves all have the same number of theoretical plates, all those eluted later than the solute which "just reaches a plateau" will reach a height lower than that acceptable for the frontal output. This intermediate case is not well described by either the Gaussian or integral Gaussian equations. Most chromatographic experiments actually fall in the class of the intermediate case, although reasonable approximations are used which often result in the use of some form of the Gaussian to describe the data.

If plate concepts are utilized and the input volume (v) is viewed as a number of infinitesimal increments, when the distribution coefficient is considered constant a series of identical profiles can be viewed as moving through the column, with each profile one plate out of phase with the one preceding it[9]. When the integral is used to approximate the summation of the solute profiles and the Gaussian is used to describe the infinitesimal elution profile, the following expression is obtained for finite inputs.

$$Y = \operatorname{erf}\left[\frac{V - V_R}{\sigma}\right] - \operatorname{erf}\left[\frac{(V - v) - V_R}{\sigma}\right] \tag{3}$$

The position of the maximum in volume units (V_{max}) may be evaluated from the derivative of eqn. 3 and eqn. 1 results. The value of Y at V_{max} is denoted Y_{max} and may be calculated by eqn. 4.

$$Y_{max} = 2\left[\operatorname{erf}\left(\frac{v}{2\sigma}\right)\right] - 1 \tag{4}$$

The width of output curves (w) resulting from infinitesimal solute inputs is roughly 4σ when defined in terms of tangents to the curves extrapolated to a baseline. For the intermediate case

$$w \approx 4\sigma + v \tag{5}$$

If the height equivalent to a theoretical plate is determined for an output curve of this type, the apparent value, H', is as in eqn. 6, in which L is the column length.

$$H' = L(w)^2/16\, V_{max}^2. \tag{6}$$

This value will be larger than the value determined for an infinitesimal input. The value can be corrected for sample volume as follows. The value of H' as $v \to 0$ is H and can be evaluated from eqn. 6a.

$$H = L(w - v)^2/16\,(V_{max} - v/2)^2 \tag{6a}$$

$$\frac{H}{H'} = \left(\frac{w - v}{w}\right)^2 \Big/ \left(\frac{V_{max} - v/2}{V_{max}}\right)^2$$

if $V_{max} \gg v/2$

$$H \approx \left(\frac{w - v}{w}\right)^2 H' \tag{7}$$

The observed resolution, R_S' of two separated curves is also affected by the input volume. Since

$$R_S' = \frac{V_{max,2} - V_{max,1}}{\frac{1}{2}(w_1 + w_2)} = \frac{V_{R,2} - V_{R,1}}{2(\sigma_1 + \sigma_2) + v} \tag{8}$$

and

$$\frac{1}{R_S'} = \frac{1}{R_S} + \frac{v}{V_{R,2} - V_{R,1}}$$

EXPERIMENTAL

Several chromatographic systems were employed in this study. One, a preparative chromatograph, consisted of a minipump (Milton-Roy)*, a loop injector (Chromatronix), and a refractive index detector (Waters Assoc.). A pressure limit switch (Barksdale) and gauge, which were inserted between the pump and injector for safety purposes, also served as a pulse dampener. Variable sample volumes were obtained by adjusting the time that the loop was switched into the mobile phase stream. A glass column (20 cm × 1 cm I.D.), dry-packed with Porasil 60A (75–125 μm diam), was employed to test eqn. 3. The solute was 0.8% solution of lactose in 0.1 M aqueous NaCl. The mobile phase was 0.1 M aqueous NaCl and the flow-rate 0.5 ml/min.

Reversed-phase chromatography of glucosyl palmitate and glucosyl stearate was performed by use of a μBondapak C_{18} column (Waters Assoc., Milford, Mass., U.S.A.; 30 × 0.4 cm) and methanol–water mixtures as mobile phase. Series of experiments were carried out on either a DuPont Model 820 liquid chromatograph which had been modified to house an air-actuated loop injector (Valco) or a Waters Series 200 liquid chromatograph fitted with the syringe-loaded, variable-volume loop injector option (Model U6K). For the former, loops of various volumes were made from precision-bore stainless-steel tubing. Refractive index detection was used in both cases. The glucosyl palmitate and glucosyl stearate were supplied by Dr. Philip Pfeffer of this Center.

RESULTS AND DISCUSSION

To test the interrelationships between input volume and output profiles, a number of chromatographic experiments were performed. Fig. 1 shows the results obtained with a preparative scale column. In chromatography, the column parameters are not known *a priori* so that a profile must be obtained first. In this case a very large feed volume was used to assure that a step function output was achieved. Then by simply measuring the difference between the height of the frontal at the de-

* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

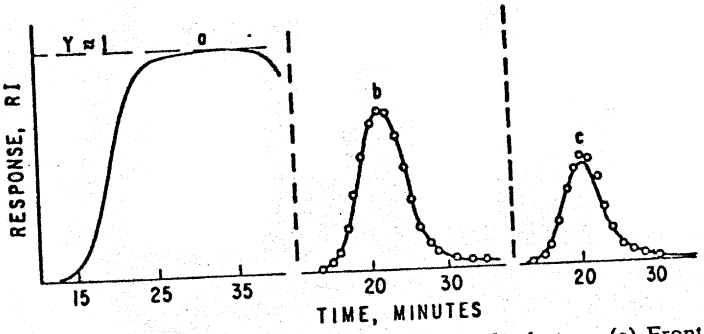

Fig. 1. Comparison of preparative LC profiles for lactose. (a) Frontal. (b) Injection time: 5 min (O) calc. from (a) using eqn. 3; (———) exp. (c) Injection time: 3 min; (O) calc.; (———) exp. See experimental section for conditions.

sired abscissa (V) and the height at ($V - v$), the ordinate value for a v ml input is obtained (eqn. 3). Any consistent system of abscissa units may be used. The experimental curves for 3- and 5-min inputs agreed well with the curves calculated from the frontal, even though the profiles were somewhat asymmetric and typical of systems in which K, the distribution coefficient, varies[10]. It is beyond the scope of this work to consider variable distribution coefficients since, when K changes with solute concentration, the superimposition concept on which the derivations are based is of limited utility. However, a useful approximation may still be obtained, particularly if the concentration range of the curve to be evaluated (for example, an intermediate case) is near to the concentration of the standard curve (for example, a frontal curve). In Fig. 1 both the 5-min and 3-min injection profiles were calculated from the frontal output curve. The agreement is better for the 5-min injection, since it is closer in concentration range to the frontal than is the 3-min injection.

A number of experiments using high-performance reversed-phase systems were designed to test the other equations herein. Typical chromatograms are shown in Fig. 2. Peak widths, w, were determined by the usual technique, and eqn. 5 was tested by plotting the data as shown in Fig. 3. The data are best fitted with straight lines by regression analysis. There is some scatter of points at small sample volumes due primarily to uncertainties in determining both the baseline and the small widths of the peaks at these low sample concentrations. These are not significant errors for the purposes of this report. The slopes of the lines are equal to the factor for conversion of the recorder chart speed to the flow-rate of eluent through the column. The slopes of the two lines differed slightly and were compared by the use of Student's t-test. The observed value of $t = 0.8848$ (28 degrees of freedom) was insignificant; thus the slopes are not significantly different from a statistical standpoint. If the average slope of 0.00284 is used, the intercepts at $v = 0$ are 3.84 and 5.92 which differ by about 2% from the intercepts of Fig. 3. Eqn. 5 is thus demonstrated to be a useful approximation, and the data should be used with no more than two significant figures.

The increase in w with input volume results in a height equivalent to a theoretical plate larger than that which would be observed at $v \to 0$. This equation was tested by converting eqn. 7 to its logarithmic form and rearranging as in eqn. 7a.

$$\log H' = \log H - 2 \log (1 - v/w) \tag{7a}$$

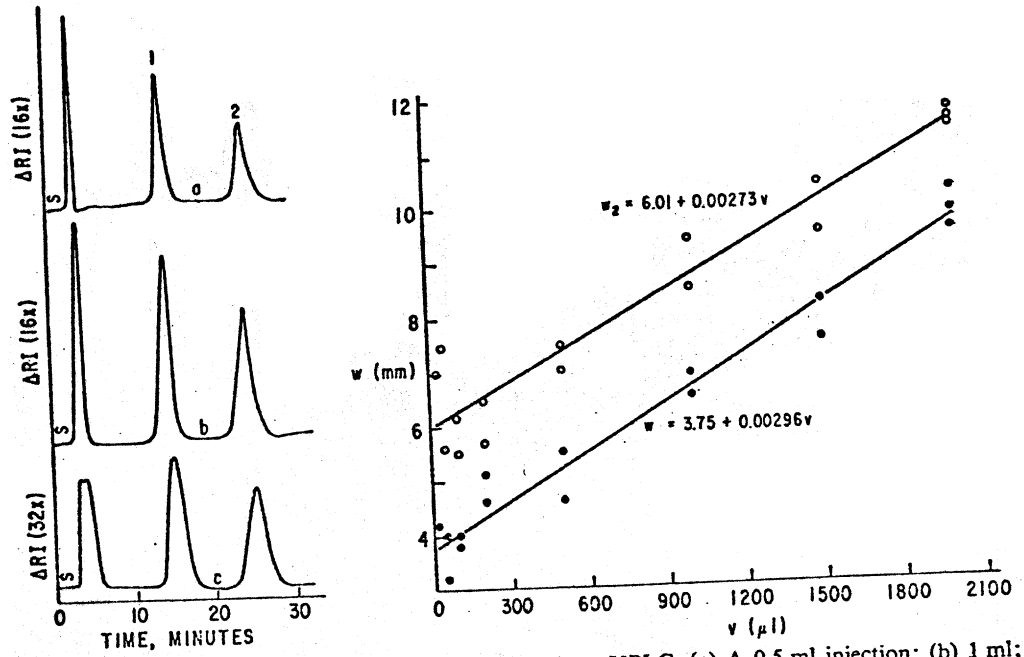

Fig. 2. Effect of large sample volumes in reversed-phase HPLC. (a) A 0.5-ml injection; (b) 1 ml; (c) 2 ml. Solute 1: glucosyl palmitate; solute 2: glucosyl stearate. Column, μBondapak C_{18}; mobile phase, methanol–water (4:1, v/v); flow-rate, 2.0 ml/min.

Fig. 3. Effect of sample volume on peak width. Conditions and solutes as in Fig. 2.

The data corresponding to that for Fig. 3 were plotted in the form of eqn. 7a. Straight lines were obtained for the data of both glucose palmitate and glucose stearate.

For the former

$$\log H' = -0.66 - 1.92 \log (1 - v/w)$$

standard error of intercept = 0.04, standard error of slope = 0.17; and for the latter

$$\log H' = -0.74 - 1.70 \log (1 - v/w)$$

standard error of intercept = 0.03, standard error of slope = 0.17.

The average slope of 1.81 agrees within about 10% with the theoretical value of 2.

The effect of increasing input volume upon peak width results in a decrease in resolution as described in eqn. 8. The information can be linearized by plotting the reciprocal of resolution as a function of v as shown in Fig. 4. The slope is the reciprocal of the difference in retention volumes at $v \rightarrow 0$, and the intercept at $v = 0$ is a function of that difference and the standard deviations (or widths) of the curves at $v \rightarrow 0$. Data for a separation of the two solutes at a slightly different eluent concentration are also given in the figure. The higher concentration of methanol in the eluent leads to a decrease in the difference between the two retention volumes; thus, $1/(V_{R,2}-$

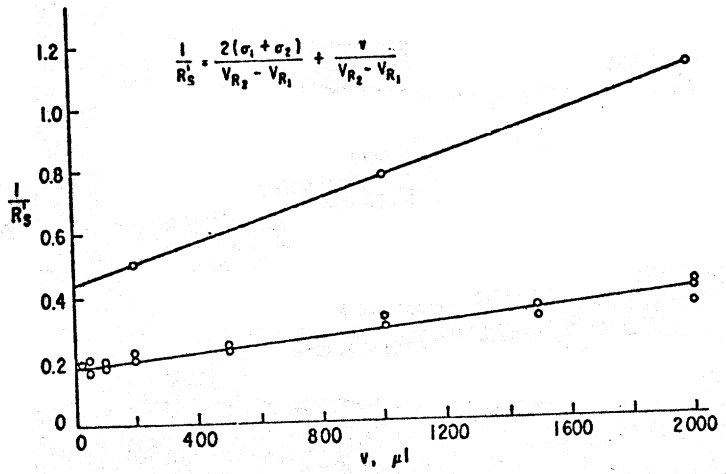

Fig. 4. Resolution–sample volume relationships. Solutes as in Fig. 2. Lower curve, conditions as in Fig. 2. Upper curve: mobile phase, methanol–water (85:15); flow-rate, 0.8 ml/min.

$V_{R,1}$) is greater for this system than for the previously discussed system. The initial resolution at $v \rightarrow 0$ is poorer and deteriorates with a higher slope, that is, is more dependent upon v. This equation may be solved for v and, for preparative purposes, the volume required to give the desired resolution may be calculated so as to optimize yield and/or purity.

A final test of the expressions is listed in Table II. The two glucose esters were fed into the column through stainless-steel loops calibrated to deliver the desired volume of solute. The data for the smallest input volume were used to calculate all the other values. Excellent agreement was found throughout.

TABLE II

COMPARISON OF CALCULATED AND EXPERIMENTAL CURVE PARAMETERS IN LIQUID CHROMATOGRAPHY FOR GLUCOSYL ESTERS

Input volume	V_{max}		*Height*		*Resolution*‡	
	*Calc.**	*Exp.*	*Calc.***	*Exp.*	*Calc.****	*Exp.*
200 µl	—	8.4	—	16.0	—	1.9
1 ml	8.9	9.1	52.0	55.0	1.2	1.3
2 ml	9.4	9.6	71.0	75.0	0.9	0.9
3 ml	9.9	9.8	78.5	78.5	0.7	0.8

* Calc. from eqn. 1.
** Calc. from eqn. 4.
*** Calc. from eqn. 8.
‡ Solvent methanol–water (85:15, v/v).

CONCLUSIONS

A straightforward relationship between sample volume and peak shape was demonstrated to exist in liquid chromatography. This relationship permits the complete description of a peak resulting from any sample volume, providing an output

curve from one known volume of input has been obtained. However, for many purposes such as estimating the point at which to collect fractions of purified solutes or choosing where to recycle when larger volumes are used, the chromatograms need only be approximated. This can be done conveniently by calculating the retention volumes, widths, heights, and resolution by use of the simpler expression contained herein.

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